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Formation of aryl and aryldiazenyl complexes in reactions of arylhydrazines and aryldiazenes with a synthetic model compound of haemoprotein

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The anaerobic reaction of chelated protohaemin, a synthetic model compound of ferrihaemoglobin, with phenyldiazene produced a compound with the visible-absorption spectrum of a ferrihaemochrome. The compound reacted with CN^-, which is a ligand of both ferric and ferrous porphyrins, to produce the complex of the synthetic ferrihaemoglobin with CN^-. Though the spectrum of the compound formed by the addition of phenyldiazene to chelated protohaemin is characteristic of a ferric porphyrin complex, this compound reacted with both toluene-*p*-sulphonylmethyl isocyanide and CO, which are strong ligands of ferrous porphyrins, to produce the corresponding ferrous complexes. These ligand-binding reactions indicated that the complex of chelated protohaem with phenyldiazene can behave either as a complex of a ferric porphyrin with phenyldiazenyl anion ($C_6H_5N{=}N^-$) or a complex of a ferrous porphyrin with phenyldiazenyl radical ($C_6H_5N{=}N^{\cdot}$). *Para* substituents on phenyldiazene were without effect on the formation of 4-substituted phenyldiazenyl complexes with chelated protohaem. *Ortho* substituents resulted in less-stable complexes. The phenyl complex of chelated protohaem was prepared by the aerobic reaction of phenylhydrazine with chelated protohaemin, and its structure was confirmed by its n.m.r. spectrum. The ligand-binding properties, n.m.r. spectrum and absorption spectrum of this complex differed from those of the phenyldiazenyl complex. The phenyl complex also was produced when the phenyldiazenyl complex was exposed to O_2.

INTRODUCTION

An aryldiazene (ArN=NH) is produced when an arylhydrazine is oxidized with 2 equivalents of ferricyanide (Itano, 1970; Mannen & Itano, 1973). Compounds with novel absorption spectra were produced when arylhydrazines were added with $K_3Fe(CN)_6$ to aqueous solutions of ferrihaemoglobin in the absence of O_2, and these compounds were postulated to be aryldiazenyl complexes of ferrihaemoglobin (Itano & Robinson, 1961; Itano & Mannen, 1976). On the other hand, when arylhydrazines were allowed to react with ferrimyoglobin, aryliron complexes of myoglobin were produced (Ringe *et al.*, 1984; Ortiz de Montellano & Kerr, 1985). Presence or absence of O_2 during the formation of aryliron complexes was not specified. Denaturation of protein interferes with spectral studies of the reaction of haemoglobin and arylhydrazines in the presence of O_2 (Itano & Matteson, 1982), and the aqueous medium complicates n.m.r. studies of solutions of haemoprotein complexes. Traylor *et al.* (1979) synthesized chelated protohaemin, a model compound that mimicked haemoglobin in its reactions with O_2 and CO. We have studied the reactions of this compound with arylhydrazines and aryldiazenes under both aerobic and anaerobic conditions. Absence of protein eliminated the problem of denaturation, and solubility in organic solvents facilitated n.m.r. studies of products.

MATERIALS AND METHODS

Phenylhydrazine hydrochloride (Aldrich Chemical Co.) was recrystallized twice from ethanol. 2-Chloro-, 4-chloro-, 2-iodo- and 4-iodo-phenylhydrazine hydrochloride (Aldrich Chemical Co.) were recrystallized from 2 M-HCl. The other ring-substituted phenylhydrazine hydrochlorides in the present study were synthesized and purified according to the methods of Itano *et al.* (1977). Protohaemin mono-3-(imidazol-1-yl)propylamide monomethyl ester [$(1)^+Cl^-$, chelated protohaemin; Fig. 1] was synthesized and purified by the methods of Traylor *et al.* (1979). The structure and the purity of the synthesized model haemoglobin were confirmed by absorption spectra, ^{1}H-n.m.r. spectra and t.l.c. (Traylor *et al.*, 1979). Toluene-*p*-sulphonylmethyl isocyanide (tosylmethyl isocyanide; $TosCH_2NC$) (Aldrich Chemical Co.) was recrystallized from ethanol. $K_3Fe(CN)_6$ (A.R.; Mallinckrodt), $Na_2S_2O_4$ (Fischer Scientific Co.), 2,6-di-t-butyl-4-methylphenol (Aldrich Chemical Co.) and other chemicals were used as obtained. O_2 was excluded in the present experiments by the use of 99.995% N_2 passed twice through a solution of vanadyl sulphate (Meites & Meites, 1948; Itano, 1970).

Reactions were conducted in a square silica cuvette of 1 cm × 1 cm cross section to which an open neck equipped with a side-arm was fused. A three-way stopcock was attached to the side-arm. N_2 was passed through the reaction mixture in the cuvette through a long needle (Hamilton). After the reactants were added and mixed under N_2, the neck of the cuvette was stoppered as the needle was withdrawn. The stopcock was then closed tightly. For reactions with CO, the gas was passed through the reaction mixture for 5 min by the method described above. $(1)^+Cl^-$ (58 μM), $TosCH_2NC$ (50 mM), phenylhydrazine hydrochloride (55 mM) and 2-

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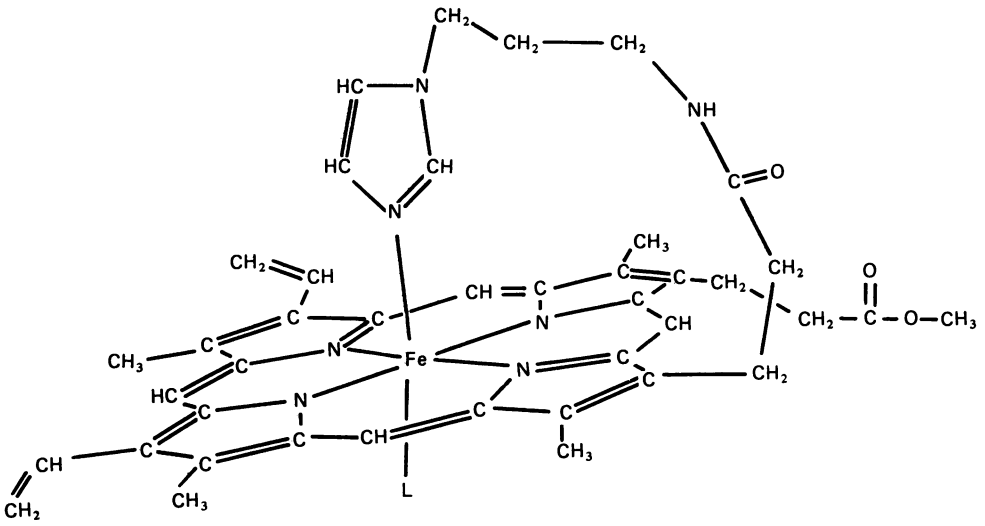


Fig. 1. Structure of chelated protohaem

This compound in its iron(III) oxidation state with L = Cl^- was synthesized and was named protohaemin mono-3-(imidazol-1-yl)propylamide monomethyl ester by Traylor *et al.* (1979). The same compound was prepared and used in the present work. Alternative designations in this paper for the compound are chelated protohaemin and $(1)^+Cl^-$. $Na_2S_2O_4$ reduces $(1)^+Cl^-$ to the unliganded iron(II) state, which is called chelated ferroprotohaem or (1) in this paper. The ligand L varies according to the reagent added and the oxidation state of the iron atom. For (1), an iron(II) compound, L = CO, $TosCH_2NC$ or CN^-, and for $(1)^+$, an iron(III) compound, L = Cl^-, CN^-, $C_6H_5N{=}N^-$ or $C_6H_5^-$, in the present work.

or 4-substituted phenylhydrazine (55 mM) in dimethylformamide were prepared under N_2.

Absorption spectra were recorded with a Cary model 17 spectrophotometer. ^{1}H-n.m.r. spectra of samples were recorded with a custom-designed 360 MHz spectrometer with 200–400 pulses in the Fourier-transform mode. Either ^2H_2O or [$^2H_7$]dimethylformamide containing tetramethylsilane were used as solvents for ^{1}H-m.m.r. spectra.

RESULTS

Reaction of (1) with $TosCH_2NC$

The addition of a 3-fold excess of $Na_2S_2O_4$ to a solution of $(1)^+Cl^-$ in dimethylformamide/water (3:1, v/v) resulted in a reddish solution, the absorption spectrum of which had $\lambda_{max.}$ at 556, 527 and 424 nm for the α-, β- and Soret absorption bands respectively of the corresponding chelated ferroprotohaem [(1); Fig. 1]. Passing CO through this solution resulted in a clear wine-coloured solution with $\lambda_{max.}$ at 566, 537 and 418 nm of the complex of (1) with CO [(1)–CO]. Addition of $K_3Fe(CN)_6$ to the solution of (1) restored the greenish solution of $(1)^+Cl^-$, which had $\lambda_{max.}$ at 600, 573 and 395 nm. These absorption spectra are shown in Fig. 2.

The stoichiometry of the reaction of (1) with $TosCH_2NC$ was investigated by the addition of 1.1-, 1.3-, 2- and 3-fold excess of the ligand to a solution of (1) generated by the reduction of $(1)^+Cl^-$ with $Na_2S_2O_4$ in dimethylformamide/water (3:1, v/v). In all of these reactions, the spectral changes occurred quickly to give the same final absorption spectrum with $\lambda_{max.}$ at 558, 529 and 428 nm as shown in Fig. 2. The reaction of (1) with equimolar $TosCH_2NC$ in [$^2H_7$]dimethylformamide and 99.96% ^2H_2O was conducted in an n.m.r. tube under N_2. The ^{1}H-n.m.r. spectrum had singlet signals at δ 0.49 and 0.25 p.p.m., which were assigned to the two protons on

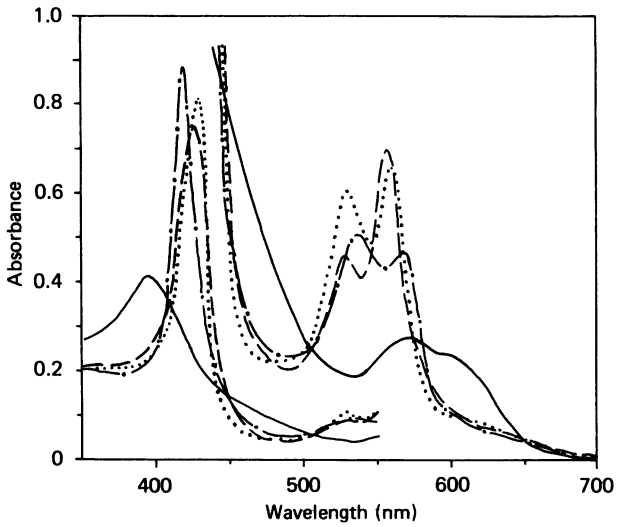
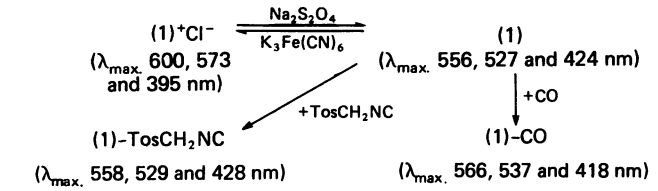


Fig. 2. Spectra of (1), $(1)^+Cl^-$, (1)–CO and (1)–$TosCH_2NC$

Visible region: into a cuvette containing 3 ml of $(1)^+Cl^-$ in dimethylformamide and 1 ml of distilled water, 4 μl of 0.13 M-$Na_2S_2O_4$ in distilled water was added. Spectra were recorded before (——) and 10 min after (— —) the addition of $Na_2S_2O_4$. The spectrum of (1)–CO (—·—) was obtained after passage of CO for 5 min through a solution of (1). The spectrum of (1)–$TosCH_2NC$ (·····) was recorded 5 min after the addition of 3.4 μl of 50 mM-$TosCH_2NC$ to the solution of (1). Soret region; concentrations were one-sixth of those used for the visible region.

the 2- and 4-positions respectively of the chelating imidazolyl group (Traylor *et al.*, 1979). Two signals at δ 7.92 and 7.55 p.p.m., which were assigned to the four protons on the *ortho* and *meta* positions of the tosyl

group of unchelated $TosCH_2NC$, were shifted to δ 5.44 and 5.12 p.p.m. when one molecule of $TosCH_2NC$ was chelated as an exogenous ligand to the iron (II) atom of a molecule of (1) to form (1)–$TosCH_2NC$. On the other hand, no spectral change was observed on the addition of $TosCH_2NC$ to a solution of $(1)^+Cl^-$ in dimethylformamide/water (3:1, v/v) other than that resulting from gradually increasing amounts of (1)–$TosCH_2NC$ attributable to the slow reduction of $(1)^+Cl^-$ by $TosCH_2NC$. Neither CO nor $Na_2S_2O_4$ changed the spectrum of (1)–$TosCH_2NC$ in dimethylformamide/water (3:1, v/v), but O_2 caused a slow change.



Reactions of $(1)^+Cl^-$ with phenylhydrazine

O_2 was excluded as described above. To 4 ml of 7.3 μM-$(1)^+Cl^-$ in dimethylformamide/water (3:1, v/v), 5 μl of 25 mM-phenylhydrazine hydrochloride, a 4.3-fold molar excess, was added. The changing spectrum of the solution was recorded periodically to obtain isosbestic points at 575, 506, 448 and 408 nm, which were the same as those of $(1)^+Cl^-$ and (1). The product obtained 90 min after the addition of phenylhydrazine was identified as (1) by its spectrum and its reactions with KCN, CO and $TosCH_2NC$. In the preceding reaction, (1) apparently was the only product. On the other hand, the spectrum of (1) was not obtained in experiments conducted at higher concentrations of the reactants by the addition of 12.6 μl of 55 mM-phenylhydrazine hydrochloride, a 4-fold molar excess, to 4 ml of 43.5 μM-$(1)^+Cl^-$ in dimethylformamide/water (3:1, v/v); spectral changes occurred without isobestic points. The final spectrum had a lower absorbance at 556 nm than that of (1), and shoulders near 565 and 530 nm replaced the peak at 527 nm (Fig. 3). To this reaction mixture, 10 μl of 0.11 M-$K_3Fe(CN)_6$ in distilled water was added to give a deep-reddish solution, the final absorption spectrum of which had $\lambda_{max.}$ at 566, 537 and 421 nm, as shown in Fig. 3. The same product (complex I) was also obtained from the simultaneous addition of phenylhydrazine and excess $K_3Fe(CN)_6$, or from the addition of a 4-fold excess of phenyldiazene generated by the oxidation of phenylhydrazine with excess of $K_3Fe(CN)_6$ in distilled water (Itano, 1970; Itano & Mannen, 1976), to a solution of $(1)^+Cl^-$ in dimethylformamide. However, no reaction was observed spectrophotometrically when a 4-fold excess of phenyldiazene was added to a solution of (1) generated by the reduction of $(1)^+Cl^-$ by $Na_2S_2O_4$. Addition of a 1.3-fold excess of $TosCH_2NC$ to a solution of complex I led to a rapid and complete change in absorption spectrum to that of (1)–$TosCH_2NC$. (1)–CO was obtained upon passage of CO through a solution of complex I. On the other hand, the complex of $(1)^+$ with cyanide $[(1)^+–CN^-]$ was formed by adding KCN to the solution of complex I as described below. Complex I was stable for more than 4 h in dimethylformamide/water (3:1, v/v) under N_2 but was very unstable in the presence of O_2. The addition of either $Na_2S_2O_4$ or $K_3Fe(CN)_6$ to a solution of complex I caused no spectral change. An attempt to isolate complex I in crystalline form was unsuccessful because of its instability towards O_2. A sample of complex I for ^{1}H-n.m.r. spectroscopy was prepared by the following procedure. A 10 μl portion of 0.21 M-phenylhydrazine hydrochloride in $[^2H_7]$dimethylformamide was added into an n.m.r. tube containing 0.25 mg of 2,6-di-t-butyl-4-methylphenol and 0.45 ml of 1.50 mM-$(1)^+Cl^-$ in $[^2H_7]$dimethylformamide under N_2. The reaction mixture was kept in the dark for 100 min, after which 150 μl of 22 mM-$K_3Fe(CN)_6$ in ^2H_2O was added under N_2. Three signals at δ 0.9, 5.9 and 6.1 p.p.m. were assigned to the aromatic protons of the ligand.

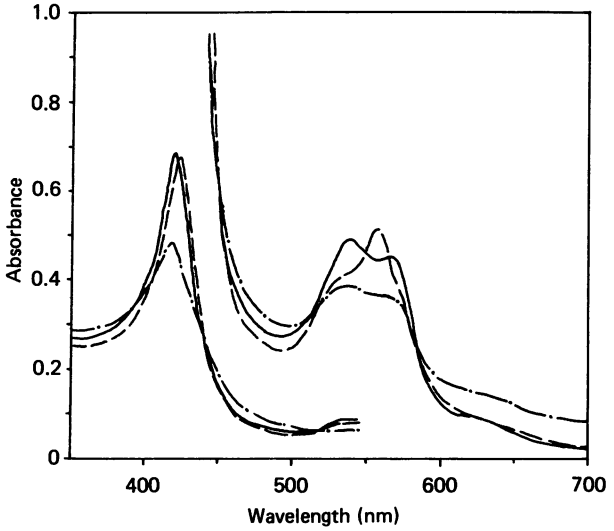


Fig. 3. Spectra of complex I and complex II

Visible region: into a cuvette containing 3 ml of $(1)^+Cl^-$ in dimethylformamide and 1 ml of distilled water, 12.6 μl of phenylhydrazine in the absence (— —) and in the presence (——) of 1.65 μmol of $K_3Fe(CN)_6$ was added. Spectra were recorded 90 min after the addition of phenylhydrazine. The spectrum (—·—) was recorded 90 min after the passing of O_2 through a reaction mixture of phenylhydrazine with $(1)^+Cl^-$ in dimethylformamide/water (3:1, v/v) containing 0.03% 2,6-di-t-butyl-4-methylphenol. Soret region: concentrations were one-sixth of those used for the visible region. ——, Complex I; (—·—), complex II.

Reactions in the presence of O_2

Passage of O_2 through a solution of complex I resulted in a reddish-brown solution. The spectra of complex I and the product had isosbestic points at 582 and 512 nm. The product (complex II) of this reaction has $\lambda_{max.}$ at 630 (shoulder), 567, 535 and 419 nm. When 25 μl of 55 mM-phenylhydrazine hydrochloride was added to 4 ml of 43.5 μM-$(1)^+Cl^-$ in dimethylformamide/water (3:1, v/v) containing 0.03% 2,6-di-t-butyl-4-methylphenol, a spectrum similar to the — — curve in Fig. 3 was recorded. Passing 500 μl of air through the reaction mixture above led to a rapid change in absorption spectrum with apparent isosbestic points at 582 and 512 nm. The spectrum of the product had $\lambda_{max.}$ at 630 (shoulder), 567, 535 and 419 nm, as shown in Fig. 3, and was identical with that of complex II. The same spectrum as that of complex II was also obtained by the

reaction of phenyldiazene with $(1)^+Cl^-$ in the presence of a small amount of O_2 in the same solvent system as above with 2,6-di-t-butyl-4-methylphenol added and by the reaction of phenylmagnesium bromide (Kunze & Ortiz de Montellano, 1983) with $(1)^+Cl^-$. The addition of a 1.3-fold excess of $TosCH_2NC$ to the solution of complex II under N_2 caused no rapid spectral change, only a gradual increase in the amount of (1)–$TosCH_2NC$. KCN reacted with complex II to give a spectrum similar to that of $(1)^+–CN^-$. A sample of complex II for ^1H-n.m.r. spectroscopy was prepared by the following procedure. A 100 μl portion of 0.2 M-phenylhydrazine hydrochloride in [$^2H_7$]dimethylformamide was added into an n.m.r. tube containing 0.25 mg of 2,6-di-t-butyl-4-methylphenol and 0.5 ml of 7.76 mM-$(1)^+Cl^-$ in [$^2H_7$]dimethylformamide under N_2. The reaction mixture was kept in the dark for 1 h; then 300 μl of air was passed into the mixture, and the n.m.r. tube was sealed. The ^1H-n.m.r. spectrum was recorded 2 h after the addition of air. Three unusual signals were found at δ −66.7, −11.8 and 22.0 p.p.m.

Reactions of $(1)^+Cl^-$ and complex I with KCN

The complex of (1) with cyanide was prepared by the addition of a 3-fold excess of KCN to a solution of (1) generated by the reduction of $(1)^+Cl^-$ with $Na_2S_2O_4$. The absorption spectrum of the complex [(1)–CN^-] had $\lambda_{max.}$ at 535 and 564 nm, as shown in Fig. 4, and was changed quickly to that of (1)–$TosCH_2NC$ by the addition of a 2-fold excess of $TosCH_2NC$. The addition of $K_3Fe(CN)_6$ to a solution of (1)–CN^- led to a rapid change in absorption spectrum to that of $(1)^+–CN^-$, having $\lambda_{max.}$ at 541 nm. The addition of $TosCH_2NC$ to $(1)^+–CN^-$ resulted in a gradual conversion into (1)–$TosCH_2NC$ over 3 h. The reaction of complex I with KCN was investigated by the addition of a 4-fold excess of KCN to a solution of the complex prepared by the reaction of $(1)^+Cl^-$ in dimethylformamide/water (3:1, v/v) with a 4-fold excess of phenyldiazene as described above. The spectrum of complex I changed rapidly to that having $\lambda_{max.}$ at 541 nm, as shown in Fig. 4; this spectrum was identified as that of $(1)^+–CN^-$.

Reactions of $(1)^+Cl^-$ with 4-substituted phenylhydrazines

Reactions of $(1)^+Cl^-$ with 4-substituted phenylhydrazines were carried out as described above with 4-chloro-, 4-iodo- and 4-t-butyl-phenylhydrazine hydrochlorides instead of with phenylhydrazine hydrochloride. These investigations led to results similar to those obtained with phenylhydrazine, i.e. the absorption spectrum of the product obtained from the reaction of $(1)^+Cl^-$ with each 4-substituted phenylhydrazine was similar to that of the — — curve in Fig. 3 in the absence of $K_3Fe(CN)_6$ and was similar to that of the —— curve in Fig. 3 in the presence of $K_3Fe(CN)_6$. The final products obtained from the reactions in the presence of $K_3Fe(CN)_6$ were also produced by the reactions of $(1)^+Cl^-$ with 4-substituted phenyldiazenes, as shown in Fig. 5. The addition of either $TosCH_2NC$ or CO to each reaction mixture containing these final products resulted in the formation of the corresponding complexes, (1)–$TosCH_2NC$ or (1)–CO, respectively. The final products were as stable as complex I in dimethylformamide/water (3:1, v/v) under N_2 and reacted minimally with either $Na_2S_2O_4$ or $K_3Fe(CN)_6$.

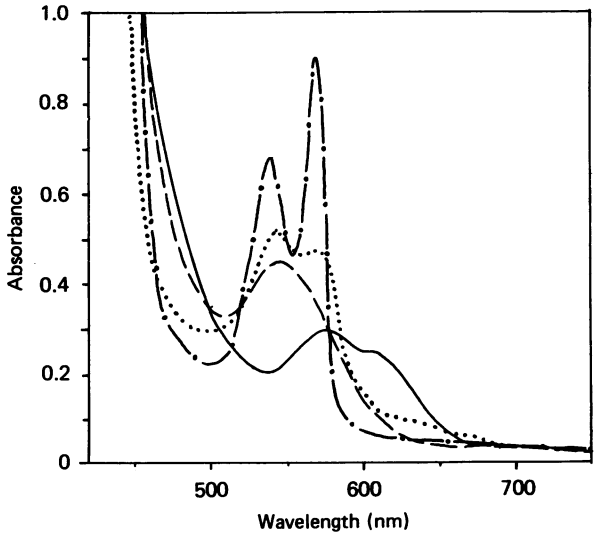


Fig. 4. Spectra of the reaction mixture of $(1)^+Cl^-$ with KCN and of (1)–CN^-

To a solution of complex I (·····) prepared by the reaction of $(1)^+Cl^-$ (——) with phenyldiazene by the same method as used for Fig. 3, 14 μl of 50 mM-KCN was added. The spectrum of the wine-reddish solution of $(1)^+–CN^-$ (— —) was recorded 2 min after the addition of KCN. The spectrum of (1)–CN^- (—·—) was recorded 2 min after the addition of KCN to the solution of (1) prepared as in Fig. 2.

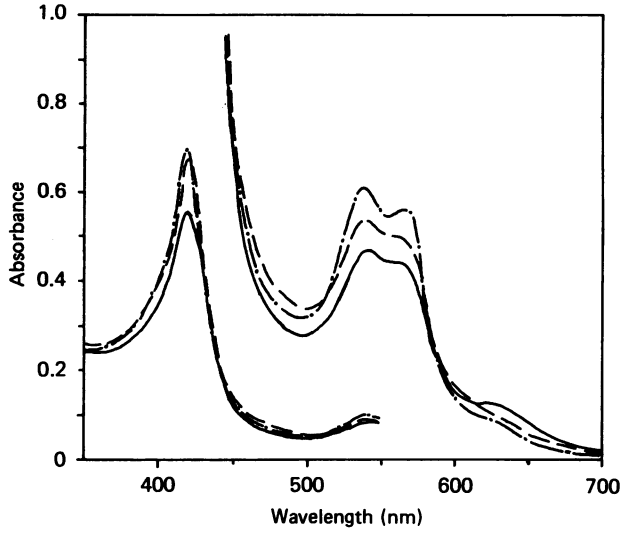


Fig. 5. Spectra of the reaction mixture of $(1)^+Cl^-$ with 4-substituted phenylhydrazine derivatives

Visible region: into a cuvette containing 3 ml of $(1)^+Cl^-$ in dimethylformamide and 1 ml of distilled water, 15 μl of 0.11 M-$K_3Fe(CN)_6$ and 12.5 μl of 4-chlorophenylhydrazine (— —), 4-iodophenylhydrazine (——) or 4-t-butyl-phenylhydrazine (—·—) were added. Spectra were recorded 90 min after the addition of each derivative. Soret region: all concentrations were one-sixth of those used for the visible region.

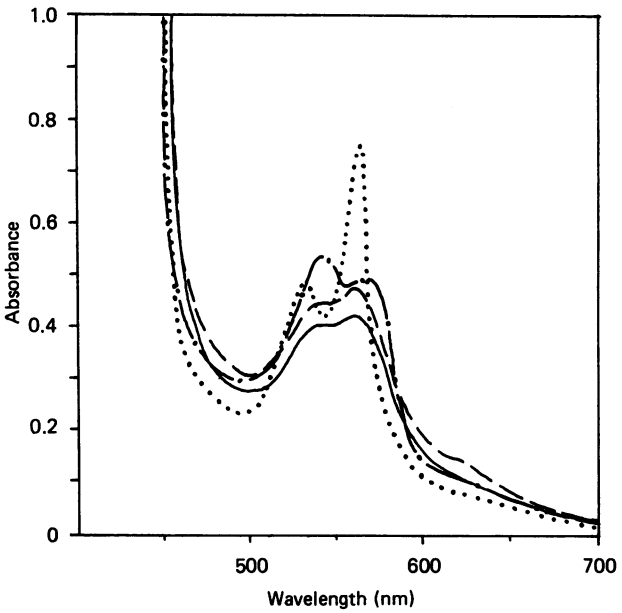


Fig. 6. Spectra of the reaction mixture of $(1)^+Cl^-$ with 2-substituted phenylhydrazine derivatives

Into a cuvette containing 3 ml of $(1)^+Cl^-$ in dimethylformamide and 1 ml of distilled water, 12.5 μl of 2-t-butylphenylhydrazine was added. The final spectrum (·····) was recorded 90 min afterwards. The spectrum (—·—) was recorded 3 h after the addition of 10 μl of 0.11 M-$K_3Fe(CN)_6$ to the reaction mixture described above. The spectra obtained from the same reactions of $(1)^+Cl^-$ with 2-chlorophenylhydrazine (— —) or 2-iodophenylhydrazine (——) were recorded 5 h after the addition of $K_3Fe(CN)_6$.

Reactions of $(1)^+Cl^-$ with 2-substituted phenylhydrazines

The reactions of $(1)^+Cl^-$ with phenylhydrazine as described above were investigated with 2-chloro-, 2-iodo-and 2-t-butyl-phenylhydrazine hydrochlorides. In the reactions at higher concentrations as well as at the lower concentrations, spectral changes with isosbestic points at 575, 506, 448 and 408 nm were observed, and all of the spectra recorded 90 min after the additions of each 2-substituted phenylhydrazine were identical with that of (1). Addition of excess of $K_3Fe(CN)_6$ to each reaction mixture described above led to slow spectral changes, the spectra of which are shown in Fig. 6. In the reaction with 2-t-butylphenylhydrazine the formation of the final complex, the spectrum of which had $\lambda_{max.}$ at 565, 537 and 419 nm, was completed over a period of 3 h after the addition of $K_3Fe(CN)_6$. The shape of the final spectrum was similar to that obtained from the reaction of $(1)^+Cl^-$ with 4-t-butylphenylhydrazine, shown in Fig. 5 as the —·— curve. In the reactions with 2-chloro- and 2-iodo-phenylhydrazine, the absorption spectrum of each complex gradually changed to a final spectrum similar to that of $(1)^+Cl^-$.

DISCUSSION

A ferrihaemochrome is defined as a low-spin iron (III) porphyrin co-ordination complex with one or more strong-field axial ligands (IUPAC–IUB Joint Commission on Nomenclature, 1980). An electronic spectrum typical of a ferrihaemochrome (Brill & Williams, 1961) appeared when phenyldiazene was allowed to react with ferrihaemoglobin (Itano & Mannen, 1976), and the product was postulated to be a co-ordination complex of phenyldiazenyl anion ($C_6H_5N{=}N^-$) with ferrihaemoglobin. A compound with a very similar electronic spectrum was produced when phenyldiazene and $(1)^+$ were allowed to react. Its optical spectrum therefore was that of a ferric haemochrome. Moreover the complex $(1)^+$–CN^- was produced by the addition of KCN to a solution of complex I. This result also indicated that the iron atom of complex I is in the ferric state. In ^{1}H-n.m.r. data for complex I, three signals at δ 0.9, 5.9 and 6.1 p.p.m. are assigned to the protons on the *ortho*, *meta* and *para* positions of a phenyl group of a phenyldiazene ligand. Although these results are consistent with the formula $(1)^+$–$C_6H_5N{=}N^-$, complex I also reacted with ligands of iron (II) porphyrins to form (1)–$TosCH_2NC$ and (1)–CO. To reconcile these observations, we postulate that a strong ligand L of iron(II) porphyrins displaces the phenyldiazenyl ligand as the radical $C_6H_5N{=}N^{\cdot}$:

$$(1)^+\text{–}C_6H_5N{=}N^- + L \rightarrow (1)\text{–}L + C_6H_5N{=}N^{\cdot}$$

The transfer of an electron from $C_6H_5N{=}N^-$ to $(1)^+$ results in the reduction of $(1)^+$ to (1) and the oxidation of the anionic ligand to a neutral radical. The displacement of $C_6H_5N{=}N^{\cdot}$ is essentially irreversible, because this radical is extremely unstable, with a mean life of only 10^{-7} s (Kasukhin *et al.*, 1974). The transfer of an electron in the reverse direction, from an iron porphyrin to its ligand, occurs in the formation of ferrihaemogloin (Hb^+) from oxyhaemoglobin (HbO_2) in the presence of an anionic ligand of Hb^+ (Wallace *et al.*, 1982):

$$HbO_2 + L^- \rightarrow Hb^+\text{–}L^- + O_2^-$$

The transfer of an electron from haemoglobin to nitrosobenzene has been postulated in the spontaneous formation of ferrihaemoglobin from nitrosobenzene–haemoglobin (Hirota & Itano, 1978):

$$Hb\text{–}C_6H_5NO \rightarrow Hb^+ + C_6H_5\dot{N}O^-$$

In the anaerobic reaction of phenylhydrazine with $(1)^+Cl^-$ at low concentrations of reactants, isosbestic points at 575, 506, 448 and 408 nm, which were the same as those between $(1)^+Cl^-$ and (1), were observed in the absence of $K_3Fe(CN)_6$; the final product was identified as (1) by its ligand-binding properties as well as its spectrum. These results indicate that phenylhydrazine reduced $(1)^+$ to (1) under the above conditions;

$$(1)^+ + \tfrac{1}{2}C_6H_5NHNH_2 \rightarrow (1) + \tfrac{1}{2}C_6H_5N{=}NH + H^+ \text{ (slow)} \quad \text{(a)}$$

On the other hand, an unusual spectrum was obtained from the reaction of phenylhydrazine with $(1)^+Cl^-$ at high concentrations of reactants in the absence of $K_3Fe(CN)_6$, as shown in Fig. 3. This spectrum is postulated to be that of the mixture of (1) and complex I produced by the rapid reaction of $(1)^+$ with phenyldiazene generated by the slow oxidation of phenylhydrazine by $(1)^+$:

$$(1)^+ + C_6H_5{=}NH \rightarrow \text{complex I (rapid)} \quad \text{(b)}$$

$$(1) + C_6H_5N{=}NH \rightarrow \text{no spectral change} \quad \text{(c)}$$

According to reaction (a), 0.5 mol of phenyldiazene is

produced per mol of $(1)^+$ reduced to (1). It is evident from this stoichiometry that the complete conversion of $(1)^+Cl^-$ into complex I in the absence of added oxidant is impossible. Not only is the maximum amount of phenyldiazene that can form insufficient, but also the formation of each mol of phenyldiazene reduces 2 mol of $(1)^+$, the other component of complex I, to (1). Some complex I forms before $(1)^+$ is completely reduced because reaction (a) is slow and reaction (b) is rapid; therefore the final solution contains both (1) and complex I.

The addition of $K_3Fe(CN)_6$ to the above reaction mixture containing (1) and complex I resulted in the conversion of (1) into complex I because remaining free phenylhydrazine was oxidized to phenyldiazene, and (1) was oxidized to $(1)^+$ (Fig. 3). These considerations are applicable to the reactions of $(1)^+Cl^-$ with 4-substituted phenylhydrazines, as *para* substituents on phenyldiazene were without effect on the formation of complexes with chelated protohaemin.

In the reactions of 2-substituted phenylhydrazines with $(1)^+Cl^-$ at both high and low concentrations in the absence of $K_3Fe(CN)_6$ spectral changes were recorded with isosbestic points at 575, 506, 448 and 408 nm; all of the final spectra obtained 90 min after the addition of each 2-substituted phenylhydrazine were identical with that of (1) (Fig. 6). These results indicated that a 2-substituted phenylhydrazine causes a reduction of $(1)^+$ to (1) as in reaction (a) but that the reactivity of $(1)^+$ towards a 2-substituted phenyldiazene is lower than that towards an unsubstituted or a 4-substituted phenyldiazene. This conclusion was supported by the fact that the formation of a complex with 2-t-butylphenyldiazene required over 3 h after the addition of $K_3Fe(CN)_6$ to the reaction mixture of 2-t-butylphenylhydrazine and $(1)^+Cl^-$ (Fig. 6), whereas complex I was formed rapidly. The slowness with incompleteness of the reactions of 2-chloro- and 2-iodo-phenylhydrazine with $(1)^+Cl^-$ in the presence of $K_3Fe(CN)_6$ and the instability of the resulting complexes indicated that 2-halogen substituents also hindered the binding of aryldiazenes to chelated haem. Hindrance by *ortho* substituents as contrasted with the absence of hindrance by *para* substituents to the binding of aryldiazenes by $(1)^+$ is consistent with the postulated model of an aryldiazenyl–haem complex (Itano, 1970) in which the N-1 atom of an aryldiazenyl ligand is bonded to the iron atom of haem.

Mansuy *et al.* (1982) and Battioni *et al.* (1983) described complexes of Fe^{II}–TPP (where TPP represents mesotetraphenylporphyrin dianion) with methyldiazene and phenyldiazene. However, we cannot compare their complexes with ours because Fe^{II}–TPP and chelated protohaem are so different structurally. Their complexes were obtained from reactions of methyldiazene or phenyldiazene with Fe^{II}–TPP as well as with Fe^{III}–TPP, whereas complex I was obtained from the reactions of aryldiazenes with $(1)^+Cl^-$, but not with (1). Moreover, their complexes have two exogenous ligands and react with $FeCl_3$ or $K_3Fe(CN)_6$ to give methyl and phenyl complexes. Complex I, in contrast, was stable in the presence of $K_3Fe(CN)_6$.

When complex I was exposed to O_2, its spectrum changed to that of complex II, with isosbestic points at 582 and 512 nm. Complex II also was produced by the aerobic reaction of phenylhydrazine with $(1)^+Cl^-$. Ringe *et al.* (1984) and Kunze & Ortiz de Montellano (1983) reported that the phenyl complex of myoglobin was prepared by the reaction of phenylhydrazine with ferrimyoglobin. In the ^1H-n.m.r. spectrum of complex II, three unusual signals were found at δ −66.7, −11.8 and 22.0 p.p.m. These signals correspond to those of the protons on the *ortho*, *para* and *meta* positions respectively of the phenyl complex of haemoproteins (Kunze & Ortiz de Montellano, 1983; Ortiz de Montellano & Kerr, 1985). The present results are consistent with the existence of both aryldiazenyl and aryl complexes of ferrihaemoproteins. The former are products of the anaerobic reaction of aryldiazene with ferrihaemoprotein. The latter are products of the aerobic reaction of arylhydrazine or aryldiazene with ferrihaemoprotein.

Doyle *et al.* (1985) obtained aryliron(III) complexes of haemoglobin ($HbFe^{III}$–Ar) in the reaction of iron(II) haemoglobin with arenediazonium salts. According to the postulated stoichiometry of their reaction, $HbFe^{III}$–Ar is a neutral species. Inasmuch as $HbFe^{III}$ (methaemoglobin) carries a positive charge, the aryl ligand of $HbFe^{III}$–Ar must carry a negative charge. We propose that complex II is an analogous compound and therefore has the formal structure $(1)^+$–$C_6H_5^-$.

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